REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (1 Jun 2003 - 31 May 2004)

4. TITLE AND SUBTITLE

EGFR Activation by Spatially Restricted Ligands

5. FUNDING NUMBERS

DAMD17-03-1-0393

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Misregulation of Transforming Growth Factor alpha (TGFalpha) and increased Epidermal Growth Factor Receptor (EGRF) activity has been associated with an increased prognosis of breast cancer. During oogenesis in *Drosophila melanogaster*, local EGFR activation by the spatially restricted TGFalpha–like ligand, Gurken (Grk), is required for axis formation in the egg and future embryo. Squid, a heterogeneous nuclear ribonucleoprotein (hnRNP) functions in the localization and translational regulation of *grk* mRNA. The purpose of this project is to identify factors that function with Squid to produce spatially-restricted EGFR activation. I found that Hrb27C, an hnRNP, interacts with Squid and plays a role in promoting *grk* localization. There is a direct interaction between Hrb27C and Ovarian tumor (Otu), a protein implicated in RNA localization. I have also gathered evidence that Squid is methylated on arginine residues. This methylation could function to mediate Squid's different roles in regulating Grk expression. I have identified an arginine methyltransferase (CG6554) that interacts with Squid, but mutations in this enzyme do not produce a phenotype during oogenesis. I am now investigating whether other methyltransferases may function in Squid methylation in the absence of CG6554. These factors could define potential causes of EGFR misregulation that result in human breast cancer.

14. SUBJECT TERMS

RNA localization, translational regulation, EGFr, TGFalpha, hnRNP, methyltransferase, genetics, molecular biology

15. NUMBER OF PAGES

24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-03-1-0393

TITLE: EGFR Activation by Spatially Restricted Ligands

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REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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INTRODUCTION

Misregulation of Transforming Growth Factor alpha (TGFalpha) and increased Epidermal Growth Factor Receptor (EGFR) activity has been associated with an increased prognosis of breast cancer (LeMaistre, 1994). TGFalpha and EGFR are required for normal breast development, but deregulation of this signaling pathway can result in uncontrolled proliferation and transformation (Matsui, 1990). I am studying the mechanism of TGFalpha activation of EGFR in the model organism *Drosophila melanogaster* in order to understand how post-transcriptional regulators of TGFalpha function to establish proper ligand expression. The purpose of this project is to identify factors that establish and maintain the tight localization of Gurken (Grk; a *Drosophila* TGFalpha-like ligand) that is necessary to produce spatially-restricted activation of the EGFR during ovary development (Schüpbach, 1987; Neuman-Silberberg & Schüpbach, 1993; 1994). These factors could define potential causes of EGFR misregulation, at the level of ligand production, that result in human breast cancer. I am integrating genetic and biochemical methods to study the effects of CG6554, Hrb27C, and Bruno on Grk expression. All three proteins interact with Squid (Sqd), a negative regulator of Grk (Kelly, 1993; Norvell et al. 1999).

BODY

The role of CG6554 in methylating Squid

The first task of the project was to determine the role of CG6554 in methylating Squid (Sqd). While I completed the initial proposed experiments, the interpretation has proven to be complicated. I found that CG6554 may function redundantly with another arginine methyltransferase to methylate Sqd. I generated multiple mutations in CG6554 and molecular characterization of these deletions suggests they are all null alleles as they remove the start codon and additional flanking sequence (Fig. 1A). In addition, western blot analysis reveals the lack of a band corresponding to CG6554 in the mutants (Fig. 1B). Interestingly, the mutants are homozygous viable and lay wild type eggs, suggesting *gurken* (*grk*) RNA and protein are properly localized. I have recombined SqdGFP onto the CG6554 mutant chromosomes and observed that SqdGFP is properly localized to the oocyte nucleus in the mutants. Immunoprecipitation of SqdGFP in the mutant backgrounds and subsequent western blot with antibodies that recognize di-methylarginine reveals that Sqd is still dimethylated in the mutant background (Fig. 2). The lack of an eggshell defect in these mutants, in addition to proper localization of *grk* RNA, Grk protein, and SqdGFP makes the fact that Sqd is still methylated not surprising.

The lack of a phenotype in the mutants caused me to see if there were any other arginine methyltransferases in the *Drosophila* genome; six additional putative arginine methyltransferases were identified with homology to CG6554 (Table 1). For one of these genes, CG5358, there was an available ovarian EST (suggesting it was expressed in the ovary) and an available P-element insertion. I obtained this P-element and generated several mutations in CG5358 by P-element excision (Fig. 3). As seen for CG6554, the mutants showed no eggshell phenotype, despite the molecular characterization of the mutants as null alleles. But, in these mutants, CG6554 is still present. It is possible that CG6554 and CG5358 function redundantly to methylate Sqd. I am currently investigating this possibility by generating flies that carry mutations in both CG6554 and CG5358. I will then examine the eggs laid by females doubly mutant for both genes and look at the state of Sqd methylation in the double mutants.

The role of Hrb27C in Gurken expression and dorso-ventral patterning

For the second task of determining the role of Hrb27C in Gurken (Grk) expression and dorso-ventral (DV) patterning, I published an article (Goodrich et al., 2004, appendix) describing

these results. Figure 2 describes the effects of *hrb27C* germline clone mutants on DV patterning. Hrb27C mutants lay eggs with a range of phenotypes that are consistent with dorsalization. These dorsalized eggs are caused by mislocalized *grk* RNA and Grk protein. Table 1 (Goodrich et al., 2004) demonstrates the in vivo genetic interaction between Hrb27C and Sqd; weak *hrb27C* mutants can strongly enhance the DV defects of weak *sqd* mutants. As Sqd binds to *grk* RNA, the interaction between Sqd and Hrb27C prompted us to ask if Hrb27C could bind directly to *grk* RNA by UV cross-linking analysis. Figure 3 (Goodrich et al. 2004) shows that Hrb27C does indeed bind to *grk* RNA. The Hrb27C antibodies do not function for whole mount staining, so I was not able to assay Hrb27C localization. However, I was able to observe that SqdGFP is properly localized in Hrb27C mutants. In addition, I observed another interesting phenotype in mutants of Sqd and Hrb27C: persistent polytene nurse cell chromosomes (Goodrich et al., 2004, Figure 4). I also found that Hrb27C interacts with another ovarian protein, Ovarian tumor (Otu). Otu seems to play a role in mediating proper Grk expression (Goodrich et al., 2004, Figure 5). Collectively, the data in this paper lead us to the model presented in Figure 6 (Goodrich et al., 2004): Sqd and Hrb27C associate with *grk* RNA in the oocyte nucleus and accompany it into the cytoplasm, where Otu then associates and other factors dissociate, to facilitate *grk* RNA localization, anchoring, and translational regulation in the dorsal anterior region of the oocyte. In the nurse cells, a distinct complex also containing Hrb27C, Sqd, and Otu in addition to other accessory factors functions in the cytoplasm to mediate the processing, localization, translational regulation, or stabilization of an unidentified RNA target, which can then regulate nurse cell chromosome dispersal at the appropriate time in oogenesis. In summary, this task was completed in full and all of the results were published.

The role of the translational regulator Bruno in Gurken expression

The third task concerned the role of the translational regulator Bruno (Bru) in Gurken expression. I have observed that Grk RNA and protein seem to be properly localized in the flies expressing only the *grk* transgene lacking all Bru binding sites in an otherwise wild type background. I have obtained a new Bruno antibody that enhances my ability to assay which sites are important for Bru binding by UV cross-linking followed by immunoprecipitation. I assayed other regions of the *grk* transcript for Bru binding and confirmed my previous results that Bru only binds the *grk* 3' UTR (Fig. 4). My current efforts are focused on this project.

KEY RESEARCH ACCOMPLISHMENTS

Task 1: To determine the role of CG6554 in methylating Squid (Sqd)

Production of 3 mutant alleles of CG6554 by imprecise P-element excision
4D8-7 (403 bp deletion), 4D8-8 (609 bp deletion), and 4C16-2 (1700 bp deletion)

CG6554 mutants lay wild type eggs

Grk RNA and protein are properly localized in CG6554 mutants

SqdGFP is properly localized in CG6554 mutants

Sqd is still methylated in CG6554 mutants

CG5358 is another methyltransferase expressed in *Drosophila* ovaries

Generation of 3 mutant alleles of CG5358 by imprecise P-element excision
2C (1718 bp deletion), 6B (845 bp deletion), and 14B (1091 bp deletion)

CG5358 mutants lay wild type eggs

Task 2: To determine the role of Hrb27C in Gurken expression and dorso-ventral patterning *this work is published in Goodrich et al., 2004

hrb27C mutants have dorsal-ventral defects

hrb27C mutants affect *grk* RNA localization and translational regulation

Hrb27C and Sqd interact genetically: weak transheterozygous alleles of Hrb27C strongly enhance the dorsal ventral defects of a weak *sqd* allele combination

Hrb27C binds to *grk* RNA

Hrb27C interacts with Ovarian tumor (Otu) by co-immunoprecipitation

otu mutants have dorsal-ventral eggshell defects

otu mutants have defects in *grk* RNA localization

Task 3: To determine the role of the translational regulator Bruno (Bru) in Gurken expression

grk RNA and protein are properly localized in the flies expressing a *grk* transgene lacking all Bru binding sites

Bru only binds to the *grk* 3'UTR, not other regions of the transcript

REPORTABLE OUTCOMES

Goodrich, J.S., Clouse, K. N., and Schüpbach, T.S. (2004). Hrb27C, Sqd, and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis. Development 131 (9), 1949-58.

Goodrich, J.S. and Schüpbach, T. (2004). The function of Squid methylation by CG6554 during *Drosophila* oogenesis. 45th Annual *Drosophila* Research Conference. Poster.

CONCLUSIONS

This work has identified Hrb27C and Otu as posttranslational regulators of Grk, which are involved in establishing proper ligand expression to achieve the spatially restricted activation of EGFR necessary for proper DV axis establishment. Mammalian homologs of these regulators can now be identified and their role in TGFalpha ligand expression can be examined as potential targets for deregulation in cancer.

CG6554 is not the only arginine methyltransferase that can methylate Sqd in the ovary and the importance of Sqd methylation is still unknown. The fact that multiple arginine methyltransferases exist and are able to methylate Sqd could imply the importance of this posttranslational modification. The future of this work should be geared toward determining the role of methylation in Sqd. As Sqd seems to be methylated in all mutants studied thus far, a transgene lacking the arginines that are presumably methylated can be generated to look at Sqd's ability to function in the absence of methylation. I will focus my efforts on this new task and look at double CG5358/CG6554 mutants. If Sqd is still methylated in the double mutants, I will look for other arginine methyltransferases that may be able to methylate Sqd. If methylation of Sqd proves to be essential for proper regulation of Grk expression, the disruption of this modification could results in improper expression of the TGFalpha ligand and ultimately misexpression of EGFR that could lead to cancer.

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Schüpbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. *Cell* 49: 699-707.

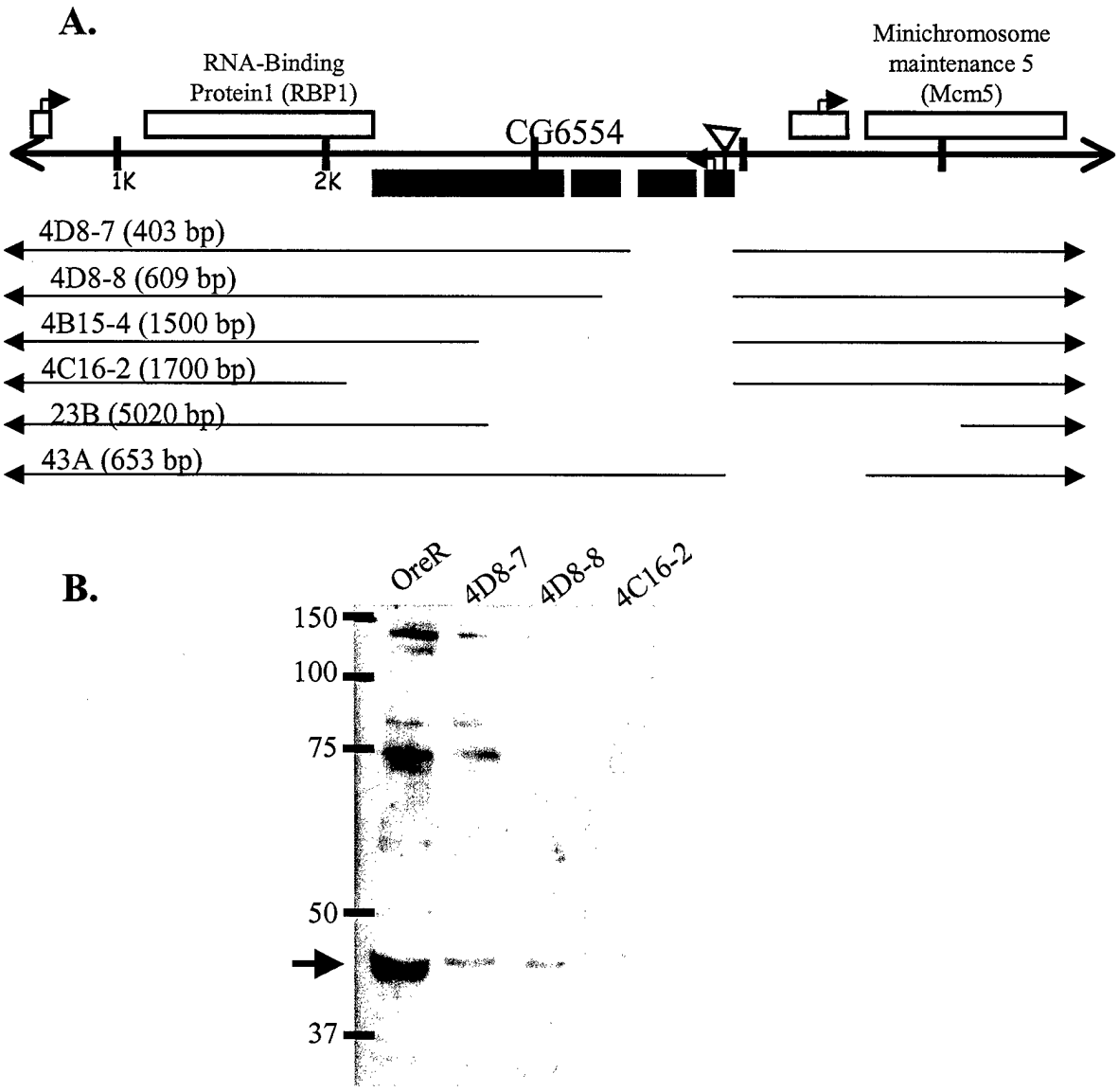

Figure 1. CG6554 mutants generated by excision analysis.
A. Schematic of the genomic region of CG6554 with a map and the names of the mutants generated by P-element excision. The deletion size is noted in parenthesis. B. Western for CG6554 protein on ovarian extract from wild type and CG6554 excision lines (4D8-7, 4D8-8, and 4C16-2) revealing the absence of the 46 kDa band, corresponding to CG6554, in the mutants. The generated mutants are null alleles.

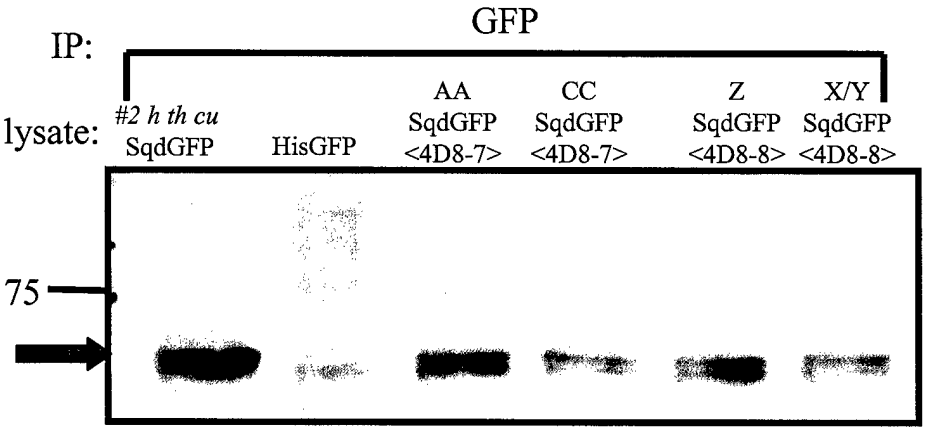

Figure 2. Sqd is still methylated in CG6554 mutants
Western blot with an antibody to dimethylated arginines (Ab 413) recognizes Sqd after GFP immunoprecipitation (IP) from ovarian extract of flies expressing SqdGFP and SqdGFP in a CG6554 mutant background. Multiple recombinants between CG6554 and SqdGFP were made and tested in this assay. There is a background band smaller than SqdGFP in the GFP IP from HISGFP ovarian lysate, but the upper band is specific to SqdGFP as seen in all the lanes. #2 SqdGFP is the line that was used to make the recombinants.

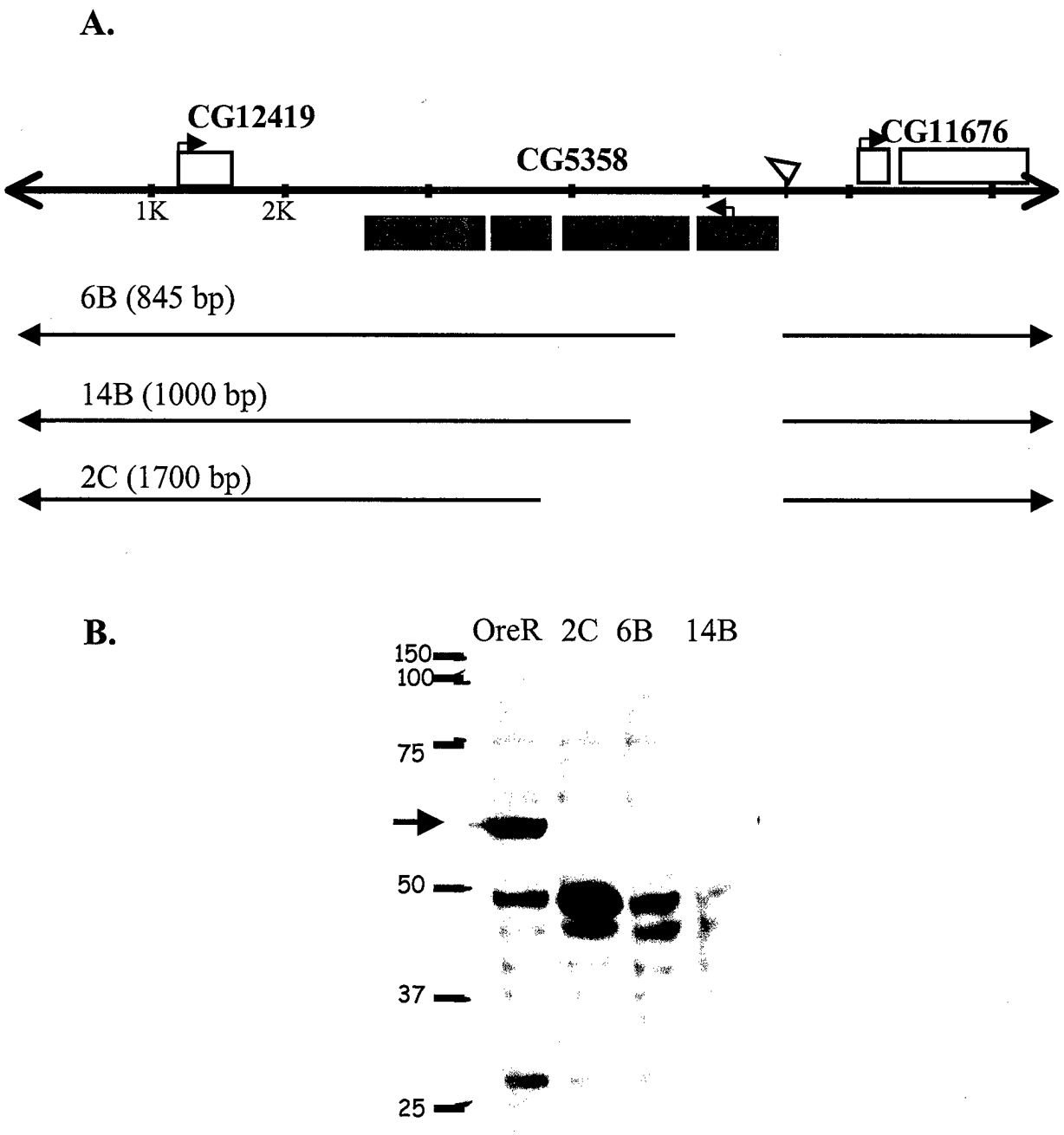

Figure 3. CG5358 mutants generated by excision analysis.
A. Schematic of the genomic region of CG5358 with a map and the names of the deletions generated by P-element excision. The deletion size is noted in parenthesis. B. Western for CG5358 protein on ovarian extract from wild type and CG5358 excision lines (2C, 6B, and 14B) revealing the absence of the 60 kDa band, corresponding to CG5358, in the mutants. The generated mutants are null alleles.

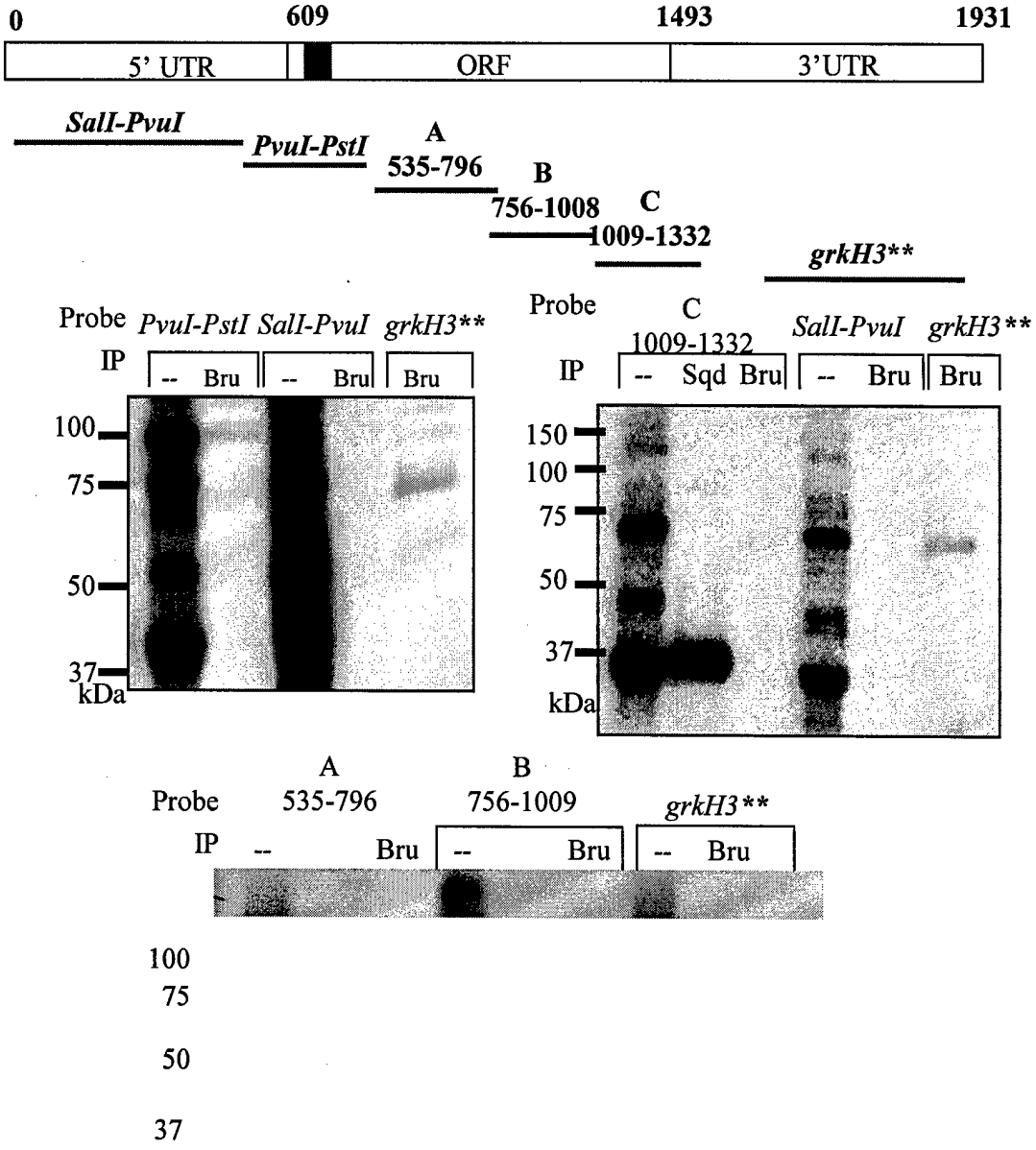

Figure 4. Bru only binds to the 3'UTR of *grk* RNA.

A. Schematic of the grk cDNA with the regions used for probes underlined. ** The region to which Bru binds.

B. UV cross-linking followed by immunoprecipitation (IP) of Bru to identify the regions of *grk* to which Bru can bind. The region used for each probe is underlined in A. In some experiments, IP with Sqd was used as a positive control.

Gene	Location	% identity to CG6554
CG6554	86C6	100%
CG3675	24E1	44%
CG6563	88E3	39%
CG9927	88A2	37%
CG5358	85F4	36%
CG16840	32D1	34%
CG9929	88A2	30%

Table 1. Seven arginine methyltransferases in the *Drosophila* genome. A list of the gene names, chromosomal location, and percent identity to CG6554 of the at least six other arginine methyltransferases in the genome.

Hrb27C, Sqd and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis

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Accepted 15 January 2004

Development 131, 1949-1958
Published by The Company of Biologists 2004
doi:10.1242/dev.01078

Summary

Heterogeneous nuclear ribonucleoproteins, hnRNPs, are RNA-binding proteins that play crucial roles in controlling gene expression. In *Drosophila* oogenesis, the hnRNP Squid (Sqd) functions in the localization and translational regulation of *gurken* (*grk*) mRNA. We show that Sqd interacts with Hrb27C, an hnRNP previously implicated in splicing. Like *sqd*, *hrb27C* mutants lay eggs with dorsoventral defects and Hrb27C can directly bind to *grk* RNA. Our data demonstrate a novel role for Hrb27C in promoting *grk* localization. We also observe a direct physical interaction between Hrb27C and Ovarian tumor (Otu), a cytoplasmic protein implicated in RNA localization. We find that some *otu* alleles produce dorsalized eggs and it appears that Otu cooperates with Hrb27C and Sqd in the oocyte to mediate proper *grk* localization. All three mutants share another phenotype, persistent polytene nurse cell chromosomes. Our analyses support dual cooperative roles for Sqd, Hrb27C and Otu during *Drosophila* oogenesis.

Key words: *Drosophila*, Oogenesis, hnRNP, Gurken, mRNA localization, Nurse cell nuclear morphology, Hrp48

Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA-binding proteins that function in a variety of cellular processes such as transcription, splicing, nuclear transport, localization, translation and protein stability (for a review, see Dreyfuss et al., 1993; Krecic and Swanson, 1999; Dreyfuss et al., 2002). HnRNP association is highly dynamic and can change with every step of RNA processing, as required by the specific RNA in the complex. Each mRNA is presumably bound by a unique combination of hnRNPs that ultimately specify its fate by influencing the structure of the RNA and modulating its interactions with trans-acting factors (Dreyfuss et al., 1993; Dreyfuss et al., 2002). Thus, hnRNPs play a crucial role in controlling gene expression.

Squid (*sqd*), also known as *Drosophila* hrp40 (Matunis et al., 1992a; Matunis et al., 1992b; Matunis et al., 1994), encodes an hnRNP that has been characterized mainly for its role in dorsoventral (DV) axis formation during oogenesis where it plays an important role in *gurken* (*grk*) mRNA localization and protein accumulation (Kelley, 1993; Norvell et al., 1999). All *Drosophila* hnRNPs identified so far have structural similarities to human hnRNP A/B proteins (Dreyfuss et al., 1993). Sqd contains the common hnRNP structural features (Krecic and Swanson, 1999; Dreyfuss et al., 2002): two RNA-binding domains, auxiliary glycine-rich and M9-like domains, and existence in multiple protein isoforms.

Grk is a TGFα-like ligand that is produced in the oocyte and signals to the EGFR (Epidermal Growth Factor Receptor) expressed in all follicle cells that comprise the epithelium surrounding the germline cells. During mid-oogenesis, establishment of the DV axis of the egg and embryo depends on the precise spatial restriction of *grk* RNA and protein to the dorsal anterior region of the oocyte to produce localized activation of EGFR (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1994). In *sqd* mutants, *grk* RNA is mislocalized and translated around the entire anterior of the oocyte producing ectopic EGFR activation and induction of excess dorsal cell fates, which results in an expansion of dorsal appendage material around the anterior circumference of the egg (Kelley, 1993). The three Sqd isoforms (A, B and S) are generated by alternative splicing and differ only in their extreme C-terminal regions. They have different subcellular localization patterns and distinct roles in the regulated nuclear export and localization of *grk* mRNA, as well as in Grk protein accumulation (Norvell et al., 1999). A working model for the role of Sqd in the regulation of Grk expression as proposed by Norvell et al. (Norvell et al., 1999) suggests that SqdS associates with *grk* RNA in the oocyte nucleus to facilitate regulated nuclear export and RNA localization within the cytoplasm, where SqdA then associates with the *grk* transcript to facilitate translational regulation. SqdB does not appear to play a role in DV patterning.

To identify proteins that interact with Sqd to function in the regulation of Grk expression, we performed a yeast two-hybrid screen with Sqd and isolated the hnRNP, Hrb27C. We found that *hrb27C* mutants have DV defects as a result of mislocalized *grk* RNA. Thus, it appears that Hrb27C and Sqd function together to regulate Grk expression. We have also identified a physical interaction between Hrb27C and Ovarian tumor (Otu). Examination of *otu* mutants reveals that *otu* also

plays a role in regulating *grk* mRNA localization. *sqd*, *hrb27C* and *otu* mutants also share a nurse cell chromosome organization defect, indicating that these proteins function together in other processes during oogenesis. mRNA biogenesis is a multi-step process, presumably involving many trans-acting factors. We report the identification of several proteins that interact with each other and regulate both *grk* RNA localization and nurse cell chromosome dynamics during oogenesis.

Materials and methods

Yeast two-hybrid screen

The two-hybrid screen was performed using the Matchmaker LexA System (Clontech). The LexA-SqdA bait was constructed by PCR amplification of a fragment from pSC1.3 (Kelley, 1993). The 5′ primer CCC CCC ATG GAA GCC GGA GAA CCA GAT G was used to add an *Nco*I site 5′ to the third exon, and the 3′ primer was T7. The resulting PCR product was cut using *Nco*I and *Xho*1 to generate a 566 bp fragment that was cloned into the pLexA vector (Clontech) to create a N-terminal fusion of LexA with amino acids 214-321of SqdA. The ovarian cDNA prey library, ovo1b, was provided by J. Groβhans (Groβhans et al., 1999) and 9.7×10^6 colonies were screened. 183 positive colonies were identified and four of these were Hrb27C.

Generation of Sqd antibodies, immunoprecipitations and western analysis

The full-length SqdA-GST fusion described in Norvell et al. (Norvell et al., 1999) was purified and used for mouse monoclonal antibody production. Immunoprecipitations were performed according to Van Buskirk et al. (Van Buskirk et al., 2000) with the following modifications: a complete mini protease inhibitor cocktail tablet (Roche) was used in lieu of other protease inhibitors in the lysis buffer, 1U/μl RNAse inhibitor (Roche) was added to the lysis buffer, lysates were not pre-cleared with pre-immune serum coated beads, and lysates were rotated with the antibody coated beads for 60 minutes at 4°C. The following antibodies were used: monoclonal anti-Sqd serum (8F3; 3:10 dilution), monoclonal anti-SpnF serum (10D8; 3:10 dilution) (U. Abdu, unpublished), polyclonal anti-Otu (guinea pig against amino acids 1-338; 1:10 dilution) (Glenn and Searles, 2001) or polyclonal anti-Odd skipped (1:10 dilution) (Kosman et al., 1998). For RNAse treated samples, 1 μg/μl RNAse A was added to the lysis buffer instead of RNAse inhibitor. NuPAGE Bis-Tris pre-cast gels (4-12%; Invitrogen) were used and the samples were transferred to nitrocellulose (Amersham) using the Xcell II blotting apparatus with standard protocol (Invitrogen), blocked in TBST (Tris-buffered saline + 0.1% Tween-20) + 5% milk + 1% BSA, incubated in anti-Hrp48 (Siebel et al., 1994) at 1:20,000 (or anti-Sqd at 1:100), washed and incubated in HRP-conjugated anti-rabbit antibody (Vector) at 1:7500 or HRP-conjugated anti-mouse antibody (Jackson ImmunoResearch) at 1:10,000. After washing, the bands were visualized by the ECL-Plus chemiluminescent system (Amersham). Germarial western analysis was performed according to Van Buskirk et al. (Van Buskirk et al., 2002) with 20 germaria in 50 μl of loading buffer (5 M Urea, 0.125 M Tris [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.1% Bromophenol Blue). Samples were loaded on a 7% NuPAGE Tris-Acetate pre-cast gel (Invitrogen), transferred, blocked, probed and detected as described above using guinea pig anti-Otu (1:500 in TBST + 5% milk) or monoclonal anti-Tubulin (1:250 in TBST + 5% milk; Sigma T-9026). HRP-anti guinea pig or HRP anti-mouse (Jackson ImmunoResearch) was used at 1:2000 in TBST.

Fly stocks

The following *hrb27C* alleles were generously provided by Don Rio and the Bloomington Stock Center: *hrb27C* [*10280, rF680, k16303, k10413, 02647, k02814*]. The following alleles are lethal P-element insertions at various distances from the coding region: $hrb27C^{10280}$ (1.5 kb), $hrb27C^{rF680}$ (1.8 kb), $hrb27C^{k16303}$ (2.2 kb), $hrb27C^{k10413}$ (2.2 kb) and $hrb27C^{02647}$ (3.3 kb) (Hammond et al., 1997). There are no molecular data on $hrb27C^{k02814}$; $hrb27C^{377}$ is an EMS allele produced by Mary Lilly. *hrb27C* FRT alleles (FRT40A-*377*, *-rF680*, *-02647*, *-10280*, *-k02814*) alleles were generously provided by Mary Lilly. The FLP-DFS (yeast flipase recombination target-site specific recombinase-dominant female sterile) system described by Chou and Perrimon (Chou and Perrimon, 1992; Chou and Perrimon, 1996) was used to generate germline clones of *hrb27C*. Progeny from *yw* hsFLP; ovoD FRT40A/ CyO × FRT40A-Hrb27C^X (*x* – one of the alleles listed above) were heat shocked at 37°C for 2 hours a day for 3 days during the second and third larval instar. The sqd^1 allele is a P-element insertion that specifically disrupts germline expression during mid-oogenesis (Kelley, 1993; Matunis et al., 1994). The isoform specific transgenes of *sqd* in a sqd^1 mutant background were described in Norvell et al. (Norvell et al., 1999). The *otu* alleles and Df*(1)RA2*/FM7, which uncovers *otu*, were obtained from the Bloomington Stock Center. hfp^9 (Van Buskirk and Schüpbach, 2002) was the allele used for RT-PCR and western analysis. Otu104 flies that carry a transgene expressing only the 104 kDa isoform of Otu under the control of the *otu* promoter (Sass et al., 1995) were provided by Lillie Searles.

In situ hybridization

Ovaries were dissected in PBS and fixed for 20 minutes in 4% paraformaldehyde in PBS with Heptane and DMSO. Subsequent steps were performed as previously described (Tautz and Pfeifle, 1989) using a *grk* RNA probe.

Immunohistochemistry

Ovaries were fixed for 20 minutes in 4% paraformaldehyde in PBS plus Heptane. After several rinses in PBST (PBS + 0.3% Triton), ovaries were blocked for 1 hour in 1% BSA + 1% Triton. After a 1 hour incubation in a 1:10 dilution of monoclonal anti-Grk sera (ID12) (Queenan et al., 1999) in PBST, the ovaries were washed in PBST overnight at 4°C. AlexaFluor 568-conjugated anti-mouse secondary (Molecular Probes) was used at 1:1000 in PBST. During secondary incubation, DNA was stained with 1:10,000 Hoechst (Molecular Probes). For DNA stain alone, ovaries were dissected and fixed as described above, incubated in 1:10,000 Hoechst for 1 hour, washed and mounted. Only stage 6 and older egg chambers were counted to assay the nurse cell phenotype.

UV cross-linking analysis

Fresh ovarian lysate was prepared according to Norvell et al. (Norvell et al., 1999) with the addition of a complete mini protease inhibitor tablet (Roche) to the lysis buffer. Radiolabeled RNA probes were made according to Norvell et al. (Norvell et al., 1999) using 1 μg of linearized (*Hin*dIII for *grk* and *osk*, *Xba*I for *nos*) DNA template. The templates for transcription include the 158 bp *Hin*cII-*Hin*dIII fragment from *grk* genomic DNA cloned into pBS, the 126 bp *Eco*RI-*Dra*I fragment from *osk* genomic DNA cloned into pBS, and the nos+6 construct (Gavis et al., 1996). Unlabelled competitor RNAs were synthesized using Ampliscribe in vitro transcription system (Epicenter) and the final product was purified by phenol extraction and ethanol precipitation. For the binding reaction, lysate equivalent to approximately three ovaries was incubated with 1.5 μl of 10× binding buffer [500 mM Tris-HCl (pH 8.3), 750 mM KCL, 30 mM $MgCl_2$], probe (5×10^5 to 1×10^6 cpm), and water to a final volume of 15 μl. The reactions were incubated for 15 minutes on ice, crosslinked on ice at 999 mJ in a Stratalinker UV crosslinker. The probe was digested for 15 minutes at 37°C with 7 units of RNAse ONE (Promega) and 1 unit of RNAse H (Roche). The immunoprecipitations were performed after RNAse digestion, by incubation of the entire reaction with antibody-coated [anti-Hrp48 or anti-CycE (Santa

Cruz#481)] protein A/G beads for 1 hour at 4°C. For the competition experiments, 200-fold excess competitor RNA was incubated with the binding reaction for 10 minutes on ice prior to addition of the probe. The concentration of competitor was determined by UV spectroscopy. After addition of loading buffer, the samples were boiled for 5 minutes, resolved on a 10% Tris-HCl Ready Gel (BioRad) and visualized by autoradiography.

Results

Hrb27C interacts with Sqd

Sqd has been shown to regulate Grk expression at the level of RNA localization and translational regulation (Kelley et al., 1993; Norvell et al., 1999). In an effort to understand the mechanism by which Sqd regulates Grk expression, a yeast two-hybrid screen with Sqd was initiated to identify interacting proteins. Using a bait containing the C-terminal region of SqdA extending from the M9-like domain to the end (Fig. 1A), we detected an interaction with four independent clones of Hrb27C. This interaction is specific (Fig. 1B) and has been confirmed by co-immunoprecipitation. Surprisingly, the association of Hrb27C and Sqd is RNA dependent; it can be disrupted by the addition of RNAse to the lysate prior to immunoprecipitation (Fig. 1C). The in vivo RNA dependence of this interaction is unexpected because the Sqd bait and at least one of the Hrb27C clones lack part of the RRMs (Fig. 1A) and are presumably not able to bind to RNA, yet the proteins are able to interact in the yeast two hybrid assay (see Discussion). Hrb27C is an hnRNP that maps to 27C and was also previously referred to as Hrp48 (Matunis et al., 1992a; Matunis et al., 1992b). Hrb27C contains two RNA recognition motifs (RRMs) (Matunis et al., 1992a) (Fig. 1A) and has been previously studied for its role in the regulation of the tissue-specific inhibition of splicing of the P-element third intron (IVS3) (Siebel et al., 1994; Hammond et al., 1997). As Sqd affects *grk* RNA localization and translational control, our findings raise the possibility that the splicing of *grk* is directly linked to localization, or, the model we favor, that Hrb27C has functions in oogenesis other than splicing.

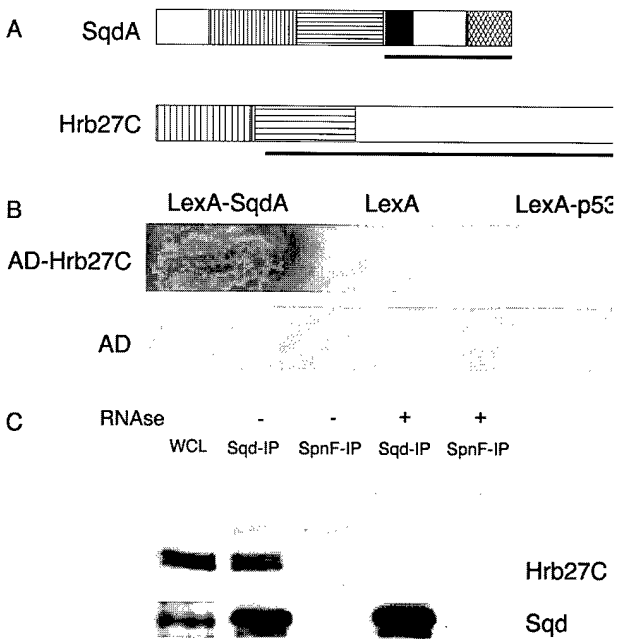

Fig. 1. Sqd and Hrb27C interact. (A) A schematic of the SqdA protein [321 amino acids (aa)] containing two RNA-binding domains (RRMs) (striped boxes), an M9-like nuclear transport motif (black box) and a glycine rich C-terminal region (hatched box). The underlined region represents the region used as bait in the yeast two-hybrid screen. The Hrb27C protein (422 aa) also contains two RNA-binding motifs (striped boxes). We isolated four different clones of Hrb27C: two contain all but the first 11 amino acids, one contains all but the first 28 amino acids and one is truncated to exclude the first 109 amino acids (underlined). (B) The Hrb27C-activation domain (AD) fusion interacts with the LexA-SqdA fusion but not with LexA alone or a LexA-p53 fusion. The AD alone does not interact with any LexA fusion proteins. (C) Western blot probed for Hrb27C and Sqd after immunoprecipitation (IP) from ovarian lysate with either Sqd or SpnF antibody in the absence (–) or presence (+) of RNAse A. Hrb27C co-purifies in an RNA-dependent manner with Sqd but not SpnF. WCL, whole cell lysate (one-tenth volume).

hrb27C mutants affect DV axis formation

To assess the effects of *hrb27C* mutations during oogenesis, we analyzed seven alleles of *hrb27C*. As *hrb27C* is an essential gene (Hammond et al., 1997), we made germline clones using the FLP-DFS system (Chou and Perrimon, 1992; Chou and Perrimon, 1996). Eggs laid by females that have Hrb27C disrupted in their germline display a variety of phenotypes, ranging from wild-type morphology in the weaker alleles to completely dorsalized eggs in the stronger alleles (Fig. 2A-D). The different classes of abnormal eggshell phenotypes are consistent with dorsalization. For two of the stronger alleles, $hrb27C^{k02814}$ and $hrb27C^{rF680}$, the observed DV defects range from being slightly dorsalized, with an expansion of the operculum (Fig. 2B), to a crown of appendage material (Fig. 2D), similar to eggs laid by strong *sqd* mutants (sqd^{1}) (Kelley, 1993). We also observed intermediate phenotypes with a broad/fused appendage that spans at least the width of the eggshell occupied by wild-type appendages (Fig. 2C). As observed in sqd^{1} mutants, most of the eggs were short, which is probably because of a failure to transfer all the nurse cell cytoplasmic contents to the oocyte prior to egg deposition.

To determine whether the observed dorsalized eggshell phenotypes of *hrb27C* are a result of defects in *grk* expression, we analyzed *grk* mRNA localization by in situ hybridization in ovaries from females whose germline was mutant for *hrb27C*. In wild-type stage 9 egg chambers, *grk* mRNA is localized to the region overlying the oocyte nucleus. Grk protein is also restricted to this same region and dorsal cell fates are induced by a local activation of EGFR in the overlying follicle cells (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1994) (Fig. 2E,G). In *hrb27C* mutants, we observed defects in *grk* RNA localization that varied depending on the strength of the allele. In the weaker mutants, all of the RNA was properly localized at stage 9, while in two of the stronger mutants, $hrb27C^{rF680}$ and $hrb27C^{377}$, 26% (n=23) and 12% (n=67), respectively, of stage 9 egg chambers showed *grk* mRNA mislocalized in an anterior ring (Fig. 2F). Thus, it seems that Hrb27C is involved in the proper localization of *grk* mRNA to the dorsal anterior region of the oocyte.

As Sqd plays a role in both RNA localization and translation

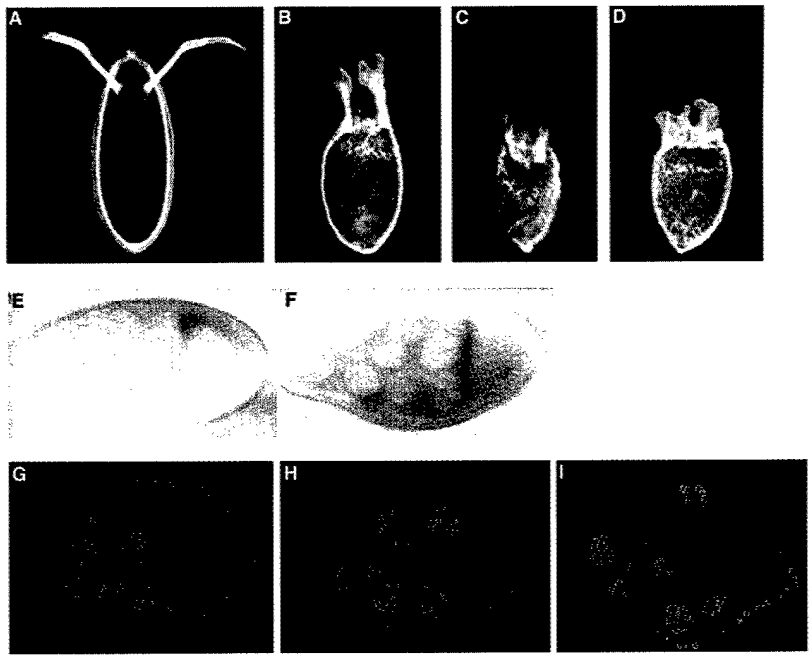

Fig. 2. *hrb27C* mutant germline clones display a dorsalized phenotype because of defects in Grk expression. (A) Wild-type egg with two dorsal appendages that mark the dorsal anterior surface. (B-D) *hrb27C* mutants lay a range of dorsalized eggs. For the two strongest alleles $hrb27C^{k02814}$ and $hrb27C^{rF680}$, the appendage defects observed range from being (B) widely spaced, with an expansion of the operculum in 28-62% of the eggs, to (D) a crown of appendage material that surrounds the anterior circumference of the egg in 5-6% of the eggs. More intermediate phenotypes were also observed (C) with a broad appendage that spans at least the width of the normal appendages in 31-60% of the eggs (n=86 for $hrb27C^{k02814}$ and n=262 for $hrb27C^{rF680}$). (E,F) *grk* in situ hybridizations of stage 9 egg chambers. In wild-type (E) *grk* is tightly localized to the dorsal anterior region of the oocyte, but is detected in a ring around the anterior circumference of a fraction of the egg chambers in *hrb27C* mutants (F). (G-I) Grk antibody staining of stage 9 egg chambers from *hrb27C* mutants reveals that Grk protein has a variable distribution that includes (G) tight localization to the dorsal anterior region as in wild-type, (H) diffuse localization throughout the oocyte with an enrichment in the dorsal anterior region, and (I) an anterior ring around the oocyte.

regulation of *grk* RNA (Norvell et al., 1999), we wanted to know if the same was true for Hrb27C. To determine if the mislocalized RNA in *hrb27C* mutants is translated, we analyzed Grk protein expression by whole-mount antibody staining. We did observe egg chambers where Grk was mislocalized throughout the oocyte cytoplasm and in a ring around the entire anterior of the oocyte (Fig. 2I), indicating that at least in some cases, mislocalized *grk* RNA is translated in the absence of Hrb27C. In many egg chambers, Grk was diffuse throughout the oocyte with an enrichment in the dorsal anterior region, but we also observed some egg chambers where Grk protein was localized normally (Fig. 2G,H). However, the occasional observation of Grk protein on the ventral side of the egg chamber clearly indicates a lack of translational repression in *hrb27C* mutants. Presumably, the egg chambers in which Grk is mislocalized in a ring would give rise to the completely dorsalized eggs laid by *hrb27C* mutant females, as this is the cause of the dorsalized eggs laid by females mutant for *sqd*.

To test whether Sqd and Hrb27C cooperate during oogenesis, we tested genetic interactions between the two genes. For this experiment, we used the few viable transheterozygous combinations of *hrb27C* alleles that produce eggs. These mutant combinations produce mildly abnormal eggshell phenotypes, which are less severe than those produced by germline clones. The hypomorphic allele sqd^{k12} also shows mild phenotypes (Kelley, 1993). sqd^{1}/sqd^{k12} transheterozygotes produce a range of mutant eggshell phenotypes, but the majority of these eggs are wild type. Mutations in *hrb27C* can enhance the eggshell phenotype of sqd^{1}/sqd^{k12}; the eggs laid by females doubly mutant for *hrb27C* and *sqd* are much more dorsalized, 18-37% of the eggs have a crown of dorsal appendage material (Table 1). This non-additive enhancement of the dorsalized phenotype is consistent with Sqd and Hrb27C cooperating in the regulation of Grk expression.

Hrb27C binds to *grk* RNA

The genetic and physical interactions of Sqd and Hrb27C suggest that these two proteins function together to regulate *grk* mRNA localization. As Sqd can bind to *grk* RNA (Norvell et al., 1999) and Hrb27C interacts with Sqd, we wanted to determine whether Hrb27C is able to bind to *grk* RNA. By performing UV crosslinking analysis in ovarian lysate using a small proportion of the *grk* 3′UTR (Fig. 3A) as a probe, we observed an interacting protein with a molecular weight (approximately 48 kDa) that is consistent with that of Hrb27C. We confirmed the identity of this protein by specifically immunoprecipitating it with Hrb27C antibodies and not with control antibodies (Fig. 3B). As a positive control, we performed the same experiments with the AB region of the *osk* 3′UTR (Fig. 3A) (Kim-Ha et al., 1995) which has previously been shown by Gunkel et al. (Gunkel et al., 1998) to bind p50, recently identified as Hrb27C (T. Yano, S. Lopez de Quinto, A. Shevenchenko, A. Shevenchenko, T. Matsui and A. Ephrussi, personal communication). Furthermore, Hrb27C

Table 1. *hrb27C* enhances the dorsalized phenotype of *sqd* mutants

	% Eggshell phenotype				
Genotype	Wild type	Fused	Wide space	Crown	*n*
sqd^{1}/sqd^{k12}	54.7	18.3	22.4	4.6	856
$hrb27C^{10280}/hrb27C^{10413}$	97.7	1.1	1.3	0.0	946
$hrb27C^{10280}/hrb27C^{10413}$; sqd^{1}/sqd^{k12}	1.4	4.4	76.7	17.6	734
$hrb27C^{10280}/hrb27C^{16203}$	97.6	0.0	2.4	0.0	742
$hrb27C^{10280}/hrb27C^{16203}$; sqd^{1}/sqd^{k12}	0.0	10.0	71.4	18.6	140
$hrb27C^{377}/hrb27C^{02647}$	1.1	0.8	98.1	0.0	795
$hrb27C^{377}/hrb27C^{02647}$; sqd^{1}/sqd^{k12}	0.0	3.7	59.3	37.0	246

The percentage of dorsalized eggshell phenotypes observed from females transheterozygous for *sqd*, transheterozygous for *hrb27C* or transheterozygous for both.

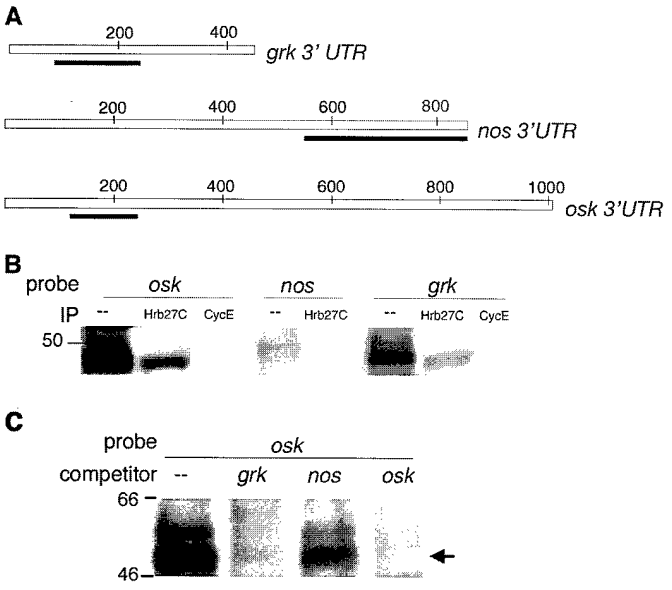

Fig. 3. Hrb27C can bind to the 3′UTR of *grk*. (A) Schematics of the entire 3′UTR of *grk*, *osk* and *nos* with the regions used as probes or competitors underlined. (B) UV crosslinking analysis of ovarian proteins that can interact with *grk*, *osk* or *nos*. The banding profiles reveal that the same proteins bind to *grk* and *osk* RNAs, but these proteins do not bind to *nos* RNA. The identity of the protein that binds to *grk* and *osk* is Hrb27C because it can be immunoprecipitated with Hrb27C antibodies, but not with CycE antibodies. (C) UV crosslinking analysis of *osk* (labeled probe) in the presence of *grk*, *osk* (positive control) or *nos* (negative control) as unlabelled competitor. A 200-fold excess *grk* or *osk* is able to compete binding of Hrb27C (arrow) from *osk*, while excess *nos* is not.

does not interact with the 3′UTR of *nanos* (*nos*) and no proteins are precipitated by the Hrb27C antibody after crosslinking with *nos* probe (Fig. 3B). Purified GST-Hrb27C can be crosslinked to the same region of the *grk* 3′UTR, while other GST fusions are not able to bind to *grk* RNA (data not shown). Finally, to demonstrate the specificity of the Hrb27C interaction with *grk* RNA and to confirm that the same protein binds to both *grk* and *osk* UTRs, we performed UV crosslinking in the presence of cold competitor RNAs. For these experiments, the 3′UTR regions of *grk*, *osk* or *nos* that were previously used as probes were added as unlabeled competitors prior to addition of the labeled *osk* 3′UTR probe. Hrb27C is completely competed away from *osk* RNA by addition of 200-fold excess *grk* 3′UTR but not by the *nos* 3′UTR (Fig. 3C). The same results were obtained using *grk* as the probe and *osk* as the competitor (data not shown). Together, these data indicate that Hrb27C can specifically bind to *grk* RNA and suggest that a complex containing Sqd and Hrb27C binds to *grk* RNA to promote RNA localization and translational regulation of Grk.

Nurse cell chromosomes of *sqd* and *hrb27C* mutants fail to disperse

We have also detected a phenotype earlier in oogenesis that is shared by both *sqd* and *hrb27C* mutants, raising the possibility that the two proteins participate in multiple processes during oogenesis. In wild-type oogenesis, the nurse cell chromosomes undergo several cycles of endoreplication that result in chromatids that are in tight association, otherwise known as polytene. Prior to their dispersal, the chromosomes take on a characteristic blob-like appearance and by stage 6, they are completely dispersed (King, 1970; Dej and Spradling, 1999) (Fig. 4A,B). In both *sqd* and *hrb27C* mutants, the nurse cell chromosomes fail to completely disperse and remain in the blob-like polytene conformation (Fig. 4C,D). Strong alleles of *sqd* (sqd^1 and germline clones of a null allele, sqd^{ix77}) have nurse cell chromosomes that are arrested in the blob-like conformation, whereas the nurse cell chromosomes of the hypomorphic allele, sqd^{k12}, disperse properly. Germline clones of all lethal *hrb27C* alleles and some of the viable transheterozygous allele combinations display some degree of polytene chromosome morphology (Fig. 4D). The sqd^1/sqd^{K12} allele combination and other viable transheterozygous *hrb27C* allele combinations ($hrb27C^{10280}/hrb27C^{k10413}$, $hrb27C^{10280}/hrb27C^{k16303}$) have nurse cell chromosomes that disperse properly. Interestingly, when sqd^1/sqd^{K12} is combined with the transheterozygous *hrb27C* allele combinations that disperse normally, the nurse cell chromosomes of the double mutants become arrested in the blob-like conformation and fail to disperse.

To determine the role of the Sqd isoforms in the dispersion of the nurse cell chromosomes, we assayed the ability of genomic cDNA transgenes that express one particular Sqd isoform (Norvell et al., 1999) to rescue the nurse cell phenotype of sqd^1 mutants. Expressing either two copies of SqdA, SqdB or SqdS in a sqd^1 background partially rescues the nuclear morphology from only 3% wild type to 14%, 54% or 61% wild type, respectively (Fig. 4E). These data suggest that each isoform is able to participate in regulating nurse cell chromosome morphology. Whereas SqdS and SqdA play the major roles in establishing the DV axis (Norvell et al., 1999), SqdS and SqdB, the two isoforms found in nuclei, have the largest effects in regulating nurse cell chromosome morphology. Consistent with the distinct yet overlapping function of the isoforms described by Norvell et al. (Norvell et al., 1999), expressing one copy of each of two different isoforms is able to rescue the nurse cell nuclear morphology better than expressing two copies of the same isoform. SqdA/SqdB rescues 96%, SqdB/SqdS rescues 96% and SqdS/SqdA rescues 92% of the nurse cell nuclear morphology to wild type (Fig. 4E).

The *ovarian tumor* gene can rescue the nurse cell nuclear morphology of *sqd* mutants

Nurse cell chromosomes that fail to disperse are also observed in certain alleles of *ovarian tumor* (*otu*) (King et al., 1981; King and Storto, 1988; Heino, 1989; Mal'ceva and Zhimulev, 1993; Heino, 1994; Mal'ceva et al., 1995). *otu* produces two protein isoforms, Otu98 and Otu104, by alternative splicing of a 126 bp exon. Genetic and molecular analyses reveal distinct requirements for each isoform during oogenesis (Storto and King, 1988; Steinhauer and Kalfayan, 1992; Sass et al., 1995; Tirronen et al., 1995). In particular, a mutant that specifically disrupts the Otu104 product has persistent polytene nurse cell chromosomes, suggesting that the 98 kDa Otu isoform is not capable of mediating wild-type chromosome dispersion (Steinhauer and Kalfayan, 1992). This phenotype was also described for mutants in *half pint* (*hfp*; *pUf68* – FlyBase). Hfp encodes a polyU-binding factor and plays an important role in the alternative splicing of *otu*.

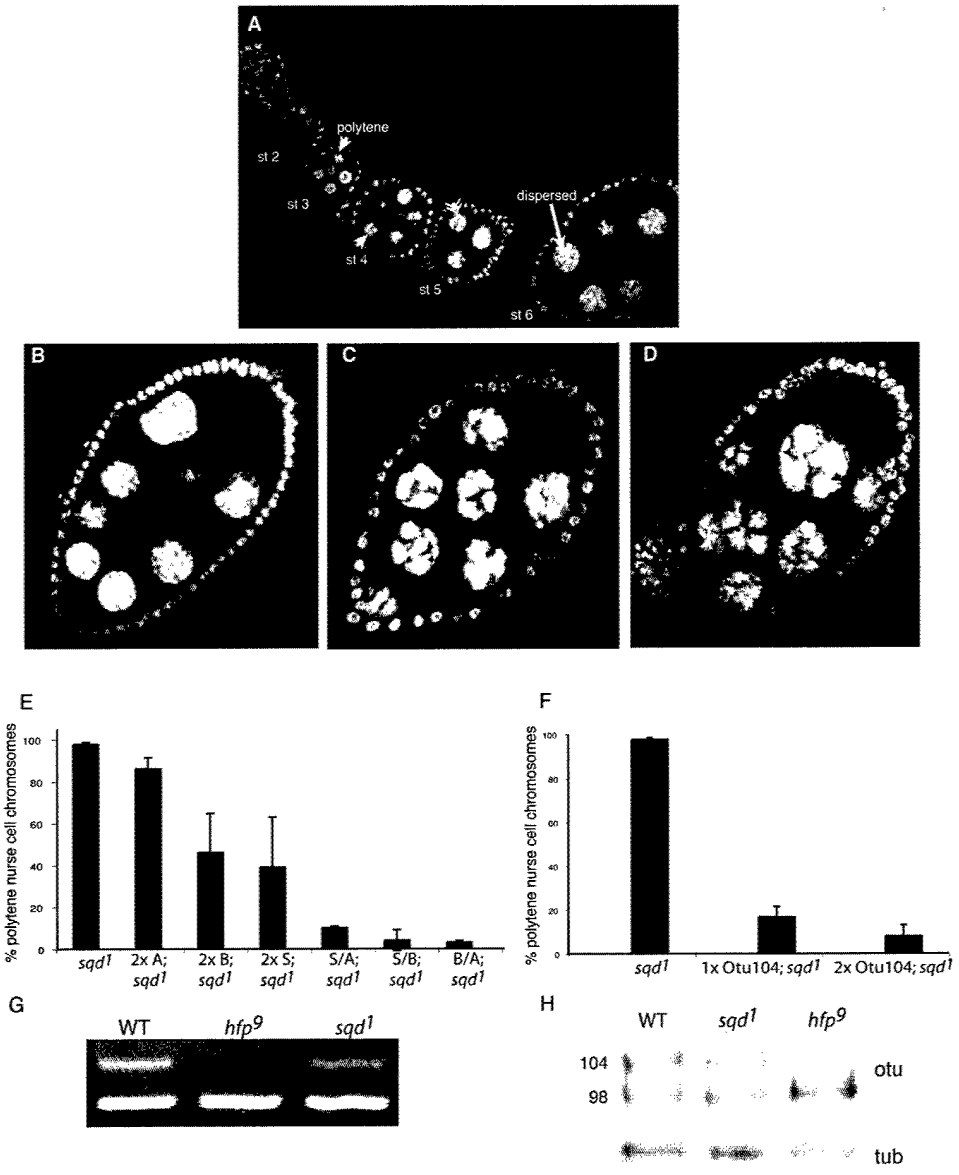

Fig. 4. *sqd* and *hrb27C* mutants have nurse cell chromosomes that fail to disperse and this phenotype is rescued by isoform-specific transgenes of Sqd and Otu. (A) Wild-type ovariole stained with Hoechst to show the polytene (arrowhead) nurse cell chromosomes that have a blob-like appearance (double arrowhead) at stage 5 and are fully dispersed (arrow) by stage 6. (B) Wild-type stage 8 egg chamber stained with Hoechst with properly dispersed nurse cell chromosomes. (C,D) *sqd* (C) and *hrb27C* (D) mutant egg chambers at stage 8 stained with Hoechst reveal that the nurse cell chromosomes fail to disperse throughout oogenesis. (E) The percentage of polytene nurse cell nuclei in stage 6 and later egg chambers from sqd^1 mutants and from sqd^1 mutants carrying transgenes expressing the specified Sqd isoforms (A, B or S) (n>400 for each genotype). (F) The percentage of polytene nurse cell nuclei in stage 6 and later egg chambers from sqd^1 mutants and from sqd^1 mutants carrying one (n=339) or two copies (n=818) of the Otu104 transgene. (G) RT-PCR analysis of *otu* transcripts in previtellarial egg chambers. Although the larger *otu* transcript is absent from *hfp* mutants, it is present in *sqd* mutants. (H) Western analysis using an Otu antibody of previtellarial egg chambers from wild type (WT), sqd^1 and *hfp* mutants. *sqd* mutants show a reduction in the amount of the 104 kDa Otu isoform. Western analysis of the samples using a Tubulin antibody reveals equal loading of the *sqd* and wild-type samples.

In *hfp* mutants, there is a dramatic decrease in the levels of the Otu104-encoding transcript as seen by RT-PCR analysis of germarial RNA, and Otu104 is not detectable on a western blot. The nurse cell phenotype of *hfp* mutants can be rescued by a transgene that expresses the 104 kDa Otu isoform under the control of the *otu* promoter (Van Buskirk and Schüpbach, 2002). To determine whether defects in Otu104 cause the polytene phenotype of *sqd* mutants, we assayed the ability of the Otu104 transgene to rescue this defect in *sqd* mutants. In sqd^1 mutants, 98% of the egg chambers (stage 6 and older) have nurse cells chromosomes that are not dispersed. When one copy of Otu104 is expressed in a sqd^1 background, only 17% of the egg chambers have persistent polytene nurse cell chromosomes, and expressing two copies of Otu104 leaves only 8% of the egg chambers with polytene nurse cell chromosomes (Fig. 4F).

As Otu104 can rescue the nurse cell phenotype of both *hfp* and *sqd*, we asked whether *sqd* affects the alternative splicing of *otu*, as is the case for *hfp* mutants. RT-PCR analysis of RNA isolated from sqd^1 germaria reveals that *otu* is properly spliced in *sqd* mutants (Fig. 4G). To determine if Otu104 protein accumulates properly in sqd^1 mutants, we performed a western blot on extracts prepared from germaria of wild-type and sqd^1 mutant females. Western analysis reveals that although both Otu isoforms are present in sqd^1; the level of the 104 kDa isoform appears reduced in sqd^1 mutants compared with wild type (Fig. 4H). Though the decrease in the levels of Otu104 observed in sqd^1 mutants is not as striking as that seen in *hfp* mutants, the decrease is consistent over many experiments. As a single copy of Otu104 can rescue the nurse cell phenotype of *sqd* mutants (Fig. 4F), and the polytene nurse cell phenotype is observed in females heterozygous for a deficiency that removes *otu* and even in females heterozygous for the Otu104-specific mutants, otu^{11} or otu^{13} (data not shown), it seems that the level of Otu104 is crucial for proper nurse cell chromosome dispersion.

Given that *hrb27C* mutants share the polytene nurse cell chromosome phenotype of *sqd* mutants, we assayed the

ability of two copies of Otu104 to rescue the phenotype of a viable transheterozygous combination of *hrb27C* (*hrb27C*^{377}/*hrb27C*^{02647}). While the Otu104 rescue of the *hrb27C* phenotype was not as complete as that of *sqd*^{1} mutants, we consistently observed a qualitatively improved (less blob-like) nuclear morphology. The Otu104-partial rescue of the nurse cell dispersion defect of *hrb27C* suggests that both Sqd and Hrb27C may act with or through Otu104 to regulate chromosome dispersal.

Otu interacts with Hrb27C and affects DV patterning

As *otu* mutants share a similar nurse cell nuclear morphology defect with *sqd* and *hrb27C*, we tested whether Otu physically interacts with these proteins. Although we have not been able to detect a physical interaction between Sqd and Otu, we do detect a physical interaction between Otu and Hrb27C by co-immunoprecipitation (Fig. 5A). This interaction is RNA independent as RNAse treatment of the lysate prior to immunoprecipitation does not disrupt it (data not shown). It is therefore possible that Otu could be part of the same complex with Sqd and Hrb27C through its interaction with Hrb27C. To evaluate a potential role for Otu in *grk* regulation, we analyzed the eggshell phenotype of eggs laid by *otu*^{7} and *otu*^{11} mutant females. Indeed, although only a few mature eggs are produced, some of these eggs are clearly dorsalized with a crown of dorsal appendage material (Fig. 5B). *otu* mutant eggs show a range of dorsalization similar to the phenotypes of *hrb27C* mutants (see Fig. 2). We also assessed *grk* RNA localization in *otu*^{7} mutants by in situ hybridization and found that *grk* RNA is mislocalized in 34% (n=92) of the stage 9 egg chambers (Fig. 5C). *grk* RNA is also mislocalized in egg chambers from *otu*^{11} females. Additional support for the role of Otu cooperating with Sqd in regulating Grk expression derives from the fact that eggs laid by females expressing two to four additional copies of Otu104 in a *sqd* mutant background have extra appendage material, but not a complete crown (data not shown). These results suggest that Otu could be in a complex with Hrb27C and Sqd that regulates *grk* RNA localization and potentially translational regulation.

Collectively, the data presented here suggest that Otu, Hrb27C and Sqd function in two different cell types in at least two processes during oogenesis. Interestingly, there seem to be differential requirements for Sqd and Otu depending on the cell type. In the nurse cells, Otu seems to be essential as expressing additional copies of Otu104 rescues the Otu104 reduction seen in *sqd* mutants (data not shown) and rescues the nurse cell chromosome morphology defect. In the oocyte, Sqd is essential for proper *grk* RNA localization. Here, extra copies of Otu104 only partially alleviate the DV defects of the *sqd*^{1} mutation. Thus, it seems that specific interacting factors make differential contributions to the coordinated function of these proteins in various cell types.

Discussion

The important roles of nuclear RNA-binding proteins in the regulation of gene expression (for a review, see Dreyfuss et al., 2002) are becoming increasingly evident as more protein complexes and their RNA targets are identified. Some hnRNPs can play multiple roles in the processing of a single RNA target; for example, in vertebrates, hnRNP A2 has been implicated in the nuclear export, cytoplasmic localization and translational regulation of myelin basic protein (for a review, see Carson et al., 2001). In *Drosophila*, the hnRNP Sqd has been shown to regulate one of its target RNAs, *grk*, at the level of localization as well as translational regulation (Norvell et al., 1999). Identification of the various partners that cooperate with Sqd should allow us to understand how one particular hnRNP can play such diverse roles in various cellular environments.

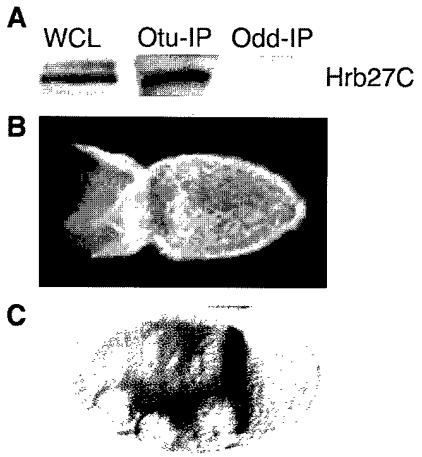

Fig. 5. Otu interacts with Hrb27C and plays a role in regulating *grk* localization. (A) Western blot probed for Hrb27C after immunoprecipitation (IP) of ovarian lysate using either Otu or Odd skipped (Odd) antibody. Only the Otu-IP shows a specific interaction. WCL, whole cell lysate (one-tenth volume). (B) A representative dorsalized egg laid by an *otu*^{7} mutant. (C) *grk* in situ hybridization of a late stage 9 egg chamber from an *otu*^{7} mutant. In wild type, *grk* is tightly localized to the dorsal anterior region of the oocyte (see Fig. 2E), but is mislocalized along the anterior circumference of the egg chamber in *otu*^{7} and *otu*^{11} mutants.

Hrb27C and Sqd interact and regulate the localization of *grk* mRNA

We have identified a physical and genetic interaction between Hrb27C and Sqd. The two proteins were previously biochemically purified as part of an hnRNP complex from *Drosophila* cells (Matunis et al., 1992a; Matunis et al., 1992b), but it was not known if they interact directly nor had their RNA targets been isolated. We originally identified the interaction in a two-hybrid screen and we have confirmed it biochemically by co-immunoprecipitation. In vivo, this interaction requires the presence of RNA; this result was unexpected because the yeast two-hybrid constructs that originally revealed the interaction did not contain the RRMs and presumably cannot bind RNA. The interaction may require the full-length proteins to be in unique conformations that are achieved only when they are bound to RNA. The truncated proteins used in the yeast two-hybrid screen may be folded in such a manner that the protein-protein interaction domains are exposed even in the absence of RNA binding, making it possible for the interaction to occur in yeast.

We also observed a striking genetic interaction between Sqd and Hrb27C; weak *hrb27C* mutants can strongly enhance the DV defects of weak *sqd* mutants. This suggests that the

physical interaction has in vivo significance. The phenotypes of *sqd* and *hrb27C* place both proteins in a pathway required for proper *grk* localization and suggest a previously undetected role for Hrb27C. *hrb27C* mutants lay variably dorsalized eggs, and in situ hybridization analysis reveals that *grk* RNA is mislocalized. As the mislocalized RNA in *hrb27C* mutants is translated, causing a dorsalized egg phenotype, it is likely that Hrb27C also functions with Sqd in regulating Grk protein accumulation. A role in the regulation of RNA localization and translation is novel for Hrb27C/Hrp48; it has been previously described as functioning in the inhibition of IVS3 splicing of P-element encoded transposase (Siebel et al., 1994; Hammond et al., 1997). Hrb27C has nuclear functions and has been observed in the nucleus and cytoplasm of somatic and germline cells in embryos (Siebel et al., 1995). As Sqd is localized to the oocyte nucleus (Matunis et al., 1994) where it is thought to bind *grk* RNA (Norvell et al., 1999), we favor a model where Hrb27C and Sqd bind *grk* RNA together and are exported possibly as a complex into the cytoplasm.

Two unpublished studies have implicated Hrb27C in *osk* RNA localization (J. Huynh, T. Munro, K. Litière-Smith and D. St Johnston, personal communication) and translational regulation (T. Yano, S. Lopez de Quinto, A. Shevenchenko, A. Shevenchenko, T. Matsui and A. Ephrussi, personal communication). Translational repression of *osk* RNA until it is properly localized is essential to prevent disruptions in the posterior patterning of the embryo (Gavis and Lehmann, 1992; Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Webster et al., 1997). An analogous mechanism of co-regulation of mRNA localization and translation appears to control Grk expression. Certain parallels between *osk* and *grk* regulation are very striking. Both RNAs are tightly localized and subject to complex translational regulation; they also share certain factors that mediate this regulation. In addition to Hrb27C, the translational repressor Bruno appears to be a part of the regulatory complexes that are required for the proper expression of both RNAs (Kim-Ha et al., 1995; Webster et al., 1997; Norvell et al., 1999; Filardo and Ephrussi, 2003). It will be interesting to determine if there are other shared partners that function in the regulation of both RNAs, as well as identify the factors that give each complex its localization specificity.

Otu plays a role in *grk* RNA localization and interacts with Hrb27C

Otu is also involved in the localization of *grk* mRNA and interacts physically with Hrb27C. Though a definitive role of Otu in regulating Grk expression has not been previously described, Van Buskirk et al. (Van Buskirk et al., 2002) found that four copies of Otu104 are able to rescue the *grk* RNA mislocalization defect of *hfp* mutants. Our analysis of the hypomorphic alleles, otu^{7} and otu^{11}, reveals a requirement for Otu in localizing *grk* RNA for proper DV patterning. Alternative splicing of the *otu* transcript produces two protein isoforms: a 98 kDa isoform and a 104 kDa isoform that differ by the inclusion of a 126 bp alternatively spliced exon (6a) in the 104 kDa isoform (Steinhauer and Kalfayan, 1992). This alternatively spliced exon encodes a tudor domain, a sequence element present in proteins with putative RNA-binding abilities (Ponting, 1997). Interestingly, the otu^{11} allele, which contains a missense mutation in exon 6a (Steinhauer and Kalfayan, 1992), shows the *grk* localization and dorsalization defect, strongly suggesting that the tudor domain of Otu plays an important function in *grk* localization. As *otu* mutants also show defects in *osk* localization (Tirronen et al., 1995), it appears that like several other factors, Otu is required for both *grk* and *osk* localization. Additionally, Otu has been isolated from cytoplasmic mRNP complexes (Glenn and Searles, 2001).

Hrb27C, Sqd and Otu function in nurse cell chromosome regulation

Surprisingly, we found an additional shared phenotype of *hrb27C*, *sqd* and *otu* mutants. During early oogenesis, the endoreplicated nurse cell chromosomes are polytene. As they begin to disperse, the chromosomes are visible as distinct masses of blob-like chromatin that completely disperse by stage 6 (King, 1970; Dej and Spradling, 1999). The formation of polytene nurse cell chromosomes is due to the endocycling that occurs during early oogenesis (Dej and Spradling, 1999) (for a review, see Edgar and Orr-Weaver, 2001). The mechanism of chromosome dispersal is hypothesized to involve the degradation of securin by the anaphase-promoting complex/cyclosome as a result of separase activity that cleaves cohesin (Kashevsky et al., 2002) (for a review, see Nasmyth et al., 2000). The significance of chromosome dispersion is not clear, but Dej and Spradling (Dej and Spradling, 1999) suggest that it could facilitate rapid ribosome synthesis. A defect in the dispersal of polytene chromosomes has been previously described for *otu* mutants (King et al., 1981; King and Storto, 1988), and we have found that both *sqd* and *hrb27C* mutants also have nurse cell chromosomes that fail to disperse. Weak double transheterozygous allele combinations of *sqd* and *hrb27C* produce the persistent polytene nurse cell chromosome phenotype that is not observed in either of the weak mutants alone. Thus, genetic and physical interactions between these gene products suggest that they function together in regulation of this process.

The failure to disperse nurse cell chromosomes has also been observed in mutants of *hfp* where the nurse cell nuclear morphology defect is due to improper splicing of Otu104 (Van Buskirk and Schüpbach, 2002). As was shown for *hfp*, expressing Otu104 in a *sqd* mutant background is able to rescue the polytene nurse cell chromosome phenotype. Although Hfp affects *otu* splicing, it is not clear how Sqd functions to mediate this phenotype, but evidence supports a role in Otu104 protein accumulation. The transcript that encodes Otu104 is clearly present in *sqd* mutants, as assayed by RT-PCR, but the level of Otu104 protein is decreased. Our results suggest that the level of Otu104 is crucial to maintaining proper nurse cell chromosome morphology as the polytene phenotype of *sqd* mutants can be rescued by expressing only one extra copy of Otu104 and the majority of egg chambers from females heterozygous for otu^{13}, otu^{11}, or a deficiency lacking *otu*, have nurse cell chromosomes that fail to disperse.

The reduced level of Otu104 in *sqd* mutants raises the possibility that Sqd could translationally regulate *otu* RNA and that the polytene nurse cell phenotype may be a result of the decreased level of Otu104. Alternatively, Sqd and Hrb27C could form a complex that recruits and stabilizes Otu104 protein, and this complex in turn functions directly or indirectly to regulate chromosome dispersion. An indirect role seems more likely, given that Otu has never been observed in the nucleus (Steinhauer and Kalfayan, 1992). As Otu104 can rescue the phenotype of *sqd* mutants and we can co-precipitate

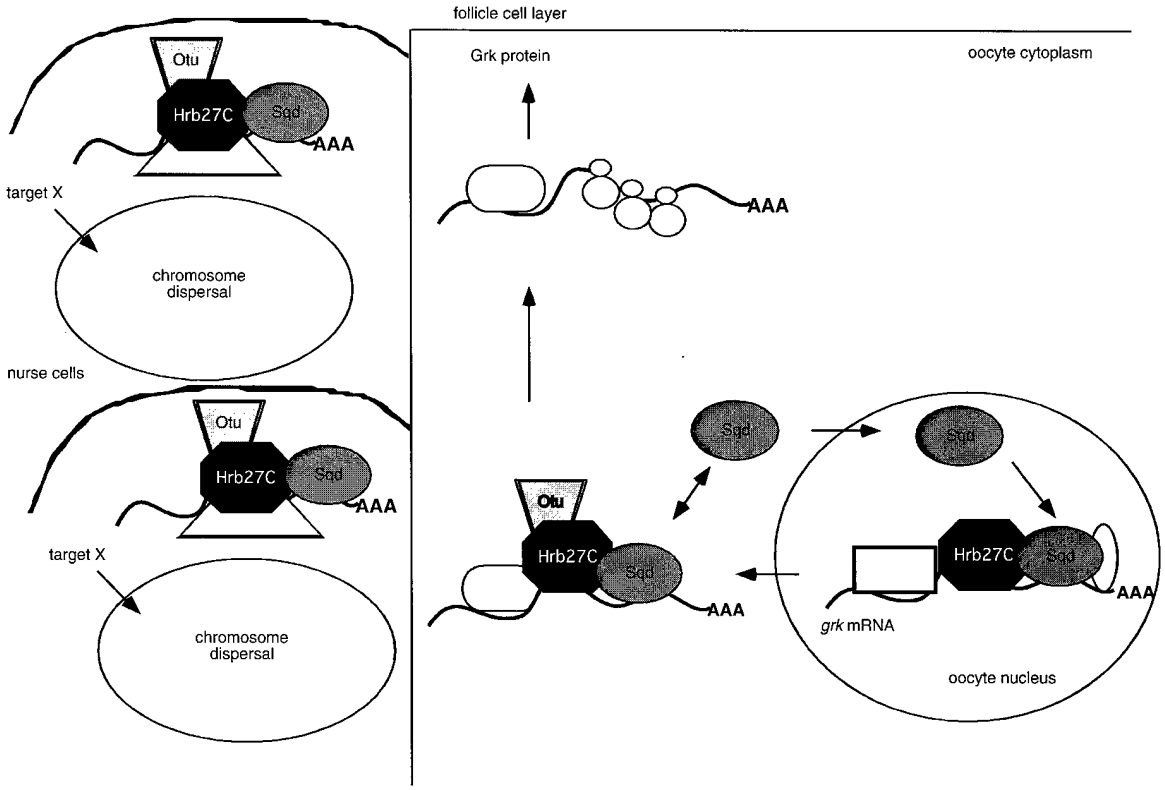

Fig. 6. Model for the dual roles of a RNP complex containing Hrb27C, Sqd and Otu in oogenesis. In the oocyte nucleus, Sqd, Hrb27C and additional factors (rectangle and oval) form a complex that binds to *grk* mRNA prior to export. Once in the cytoplasm, Sqd and Hrb27C remain part of the complex, some factors dissociate and Otu, along with different accessory factors (rounded rectangle), associate to form a distinct complex to facilitate *grk* RNA localization, anchoring and translational regulation in the dorsal anterior region of the oocyte. In the nurse cells, a distinct complex also containing Hrb27C, Sqd and Otu in addition to other accessory factors (trapezoid) function in the cytoplasm to mediate the processing, localization, translational regulation or stabilization of an unidentified RNA target (X), which can then regulate nurse cell chromosome dispersal at the appropriate time in oogenesis.

Otu and Hrb27C, it seems plausible that Sqd and Hrb27C interact with Otu in the nurse cell cytoplasm to affect an RNA target that could then mediate chromosome dispersion.

Model for a RNP complex containing Sqd, Hrb27C, and Otu

Our genetic data support a model in which Sqd, Hrb27C, and Otu function together in a complex that affects at least two processes during oogenesis: DV patterning within the oocyte and mediation of nurse cell chromosome dispersion. Although our biochemical data are consistent with this model, it is also possible that Hrb27C and Sqd could form a complex that is distinct from a complex containing Hrb27C and Otu. However, the in vivo genetic interactions and mutant phenotypes reveal that all three proteins affect both processes and lead us to favor a model in which all three proteins function in one complex.

Our results allow us to expand upon the model proposed by Norvell et al. (Norvell et al., 1999) for the regulation of *grk* RNA expression (Fig. 6). Sqd and Hrb27C associate with *grk* RNA in the oocyte nucleus. Hrb27C and Sqd remain associated with *grk* in the cytoplasm where Otu and possibly other unidentified proteins associate with the complex as necessary to properly localize, anchor and translationally regulate *grk* RNA. A likely candidate to be recruited to the complex is Bruno, which interacts with Sqd (Norvell et al., 1999). Although we do not know the RNA target of Sqd, Hrb27C and Otu in the nurse cells, the proteins could form a complex composed of different accessory factors to regulate localization and/or translation of RNAs encoding proteins that affect chromosome morphology.

Although Hrb27C, Sqd and Otu function together to affect different processes in the oocyte and the nurse cells, they probably function to mediate the precise spatial and temporal regulation of a target RNA that is unique to each cell type where the presence of additional factors would provide specificity to each complex. The importance of the precise localization and translational regulation of *grk* is well defined, and we have now identified Hrb27C and Otu as additional proteins that facilitate these processes. The transition from polytene to dispersed chromosomes in the nurse cells is less well characterized, but most probably requires precise spatial and temporal regulation as well. A complex containing Sqd, Hrb27C and Otu could function to ensure that the target RNA is properly localized and functional in the nurse cells only at the proper stage of development to promote chromosome dispersal.

We thank Don Rio for providing the Hrp48 antibody and *hrb27C* mutant fly stocks; Lillie Searles for the Otu antibody and Otu transgenic flies; Mary Lilly for providing the *hrb27C* FRT fly stocks and communicating unpublished data; Jörg Großhans for ovarian cDNA library; and Liz Gavis for the nos+6 construct. We also thank

Anne Ephrussi and Daniel St Johnston for communicating results prior to publication. We are grateful to Gail Barcelo for help with in situ hybridizations, and to Uri Abdu, Cheryl VanBuskirk and Amanda Norvell for enlightening discussions throughout the course of the project. Thanks to Andrew Swan, Cheryl VanBuskirk and Amanda Norvell for critical reading of the manuscript; Joe Goodhouse for assistance with confocal microscopy; and Marty Marlow for monoclonal antibody production. This work was supported by the Howard Hughes Medical Institute and US Public Health Service Grant PO1 CA41086. J.S.G. is supported by the DoD Breast Cancer Research Program DAMD17-03-1-0393.

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